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THE DIFFERENTIATION OF BACTERIA OF THE COLON-AEROGENES FAMILY BY THE USE OF INDICATORS *

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INTRODUCTION

It is a rare pleasure to see an old scientific deduction re-established after a period of abuse and rejection. Dr. Theobald Smith discerned in the differences of gas production among bacteria of the colon-aerogenes family a means of differentiation. That distinct differences exist has not been seriously disputed; but the methods of detecting them were left with no valuable improvements for nearly twenty years. During this time an enormous mass of inaccurate data accumulated and produced a confusion from which it was impossible to extricate conclusions sufficiently clean-cut to force conviction. Consequently, the gas ratio was rejected as untrustworthy, the Smith fermentation tube, after the exhaustion of its value as an instrument for pioneer research, was relegated to its proper position, and the more fundamental questions which its use had raised were to a large degree forgotten.

Then Keyes[1] pointed out the reasons for the hopeless inaccuracies of the fermentation tube and developed an accurate method for the collection of the gases. With slight improvements upon this method, Rogers, Clark, and Davis[2] found that they could obtain results which furnished an admirable basis for the differentiation of the colon-aerogenes group.

In the three investigations now completed by Rogers, Clark, and Davis[2] and Rogers, Clark, and Evans,[3] the fundamental deduction of Theobald Smith upon the value of the gas ratio has been abundantly confirmed. It has been shown that bacteria of the colon-aerogenes family can be sharply divided into two groups on the basis of the gas ratio. When grown in vacuo in a medium consisting of 1 percent Witte peptone, 1 percent dextrose, and 0.5 percent K_2HPO_4, one group produces

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[1] Jour. Med. Research, 1909, 21, p. 69.
[2] Jour. Infect. Dis., 1914, 14, p. 411.
[3] Ibid., 15, p. 99; Ibid., 1915, 17, p. 137.

carbon dioxid and hydrogen in the constant ratio $CO_2/H_2=1.06$. Members of this group have been termed "the low ratio organisms." The second group produces much more carbon dioxid than hydrogen, and furnishes a ratio which varies from about 1.90 to 3.00. Such bacteria are called members of "the high ratio group."

In the experimental work referred to, there were encountered a few organisms which produce carbon dioxid but no hydrogen. They are distinguished by certain cultural characteristics which set them apart from the colon-aerogenes family, but they will be treated in the following discussion as a third group. Since they liberate no hydrogen, the ratio $CO_2:H_2$ becomes infinity. We shall retain the term "∞ ratio group" as a convenient means of designation.

Every advance made in the study of these three groups of gas-producing bacteria has added force to the belief that the gas ratio is an index to certain fundamental differences in metabolism. Consequently, when accurately determined, it may be depended upon to furnish a logical basis for the preliminary separation into main groups. Such use of it was made in the three investigations to which reference has been made. In the results there appeared a remarkable correlation between the gas ratio and the source of the cultures, which is summarized in the following table:

TABLE 1

CORRELATION BETWEEN GAS RATIO AND THE SOURCE OF THE CULTURE

Source	Total Number of Cultures	Number of Low Ratio Cultures	Number of High Ratio Cultures	Number of ∞ Ratio Cultures
Milk and milk products.	124	65	59	1
Bovine feces	150	149	1	0
Grain	166	8	151	7

The sanitary significance of this correlation is perfectly obvious. But it is also clear that the methods by which the gas ratio can be accurately determined are not adapted to the routine examination of large numbers of cultures. This leaves certain laboratories in a rather embarrassing position, for while the sanitary significance of the gas ratio may be appreciated, no method applicable to routine work is available. It is therefore imperative that some characteristics be found which will correlate accurately with the gas ratio, and which may be accurately and easily distinguished. By taking cognizance of certain characteristics of the cultures at hand, the desired correlation has been found, and a simple method of diagnosis worked out. This method can best be presented by showing its logical development.

DEVELOPMENT OF THE METHOD

In 1912 Michaelis and Marcora[4] showed that a certain culture of Bacillus coli, when grown in a lactose broth, ceased activity at a hydrogen ion concentration of 1×10^{-5} N (this term being an abbreviation for the equivalent of 0.00001 gm. H per liter). This point is reached independently of the initial reaction of the medium. Michaelis and Marcora considered it to be a physiologic constant for Bacillus coli.

Since these authors apparently worked with but one culture and one medium, it remained to be determined whether this limiting hydrogen ion concentration is a constant for other cultures of Bacillus coli and in media of such widely varying composition that it could truly be called a characteristic physiologic constant. Such a research has been progressing for two years and during this time so many interesting facts have been observed that publication has continuously been delayed pending "the next set of experiments." In view of the fact that certain of the results have proved to be of immediate practical value in solving the problem at hand, they may be sketched as follows:

All organisms of the colon-aerogenes family which give a low gas ratio will, if furnished sufficient fermentable carbohydrate, continue to elaborate acid until a certain zone of hydrogen ion concentration is reached. There all activity ceases. The particular point in this zone is determined by the nature of the medium; but in any given medium the particular point reached by a variety of cultures is remarkably constant. In a medium such as that used by Michaelis and Marcora, the point is very close to 1×10^{-5}, as these authors found. With simpler media, containing dextrose, the point reached is appreciably higher, as Table 2 will show.

TABLE 2

FINAL HYDROGEN ION CONCENTRATION OF "LOW RATIO CULTURES"*

Organism	$C\overset{+}{H}$
22 fg	4.8×10^{-5}
22 fg	4.7×10^{-5}
22 hw	4.9×10^{-5}
22 hw	5.0×10^{-5}
22 hx	4.8×10^{-5}
22 hy	4.8×10^{-5}
22 hy	5.1×10^{-5}
22 hz	4.7×10^{-5}
22 hz	4.9×10^{-5}

* The medium consisted of 1 percent Witte peptone and 1 percent dextrose in Jena test tubes. Incubation was maintained at 30 C. for a period of fifteen days.

In determining hydrogen ion concentrations, the electrometric method has been used. The chain employed was the customary one: Hg HgCl | N/10 KCl | N/10 KCl | Sat. KCl | Solution | H_2 Pt. The electrode vessel was a special form based upon the principles of Hasselbalch's[6] improved design. This and other details will be described elsewhere.

On the other hand, cultures of the "high ratio organisms," in media in which the "low ratio organisms" reach their limiting hydrogen ion concentration, are found to reach much lower values. This is shown in Table 3. The gas ratios included in this table are those given in the papers of Rogers, Clark and Evans.[3]

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[4] Ztschr. f. Immunitätsforschung, 1912, 14, p. 170.

[5] Biochem. Ztschr., 1913, 49, p. 433.

TABLE 3

HYDROGEN ION CONCENTRATIONS OF "LOW" AND "HIGH RATIO CULTURES"* COMPARED

Organism	Ratio $CO_2: H_2$	$C\overset{+}{H}$	Organism	Ratio $CO_2: H_2$	$C\overset{+}{H}$
22 hx	1.03	2.8×10^{-5}	22 ty	2.60	7.3×10^{-7}
22 hz	1.06	3.3×10^{-5}	22 sc	2.59	9.0×10^{-7}
			22 se	2.08	6.7×10^{-7}
22 om	2.28	6.0×10^{-7}	22 nd	2.22	6.3×10^{-7}
22 hg	2.44	5.1×10^{-7}	22 ni	2.52	1.0×10^{-7}
22 rq	2.36	7.0×10^{-7}	22 nn	2.24	5.7×10^{-7}
22 rr	2.24	5.1×10^{-7}	22 no	2.18	5.7×10^{-7}
22 rs	2.44	7.0×10^{-7}			
22 rt	2.34	1.6×10^{-6}			

* The medium consisted of 1 percent Witte peptone and 1 percent dextrose, 0.050 Molecular H_3PO_4; 0.075 Molecular Na OH. The incubation temperature was 30 C. for a period of seven days. Cultures were held in Jena test tubes.

It must not be inferred from this that the "high ratio organisms" cease fermentation at such low hydrogen ion concentrations because they can endure none higher. Rather does it appear that these organisms have fermented all the sugar with the production of a quantity of acid insufficient to inhibit further activity. If the dextrose content of the medium be increased, it is found that, up to a certain concentration of sugar, the gas produced is increased and the final hydrogen ion concentration is raised. This is shown in Table 4.

TABLE 4

GAS PRODUCTION OF "HIGH RATIO ORGANISMS"*

Organism	Percentage of Sugar	Total Gas in c.c.	Ratio $CO_2: H_2$	Final $C\overset{+}{H}$ of Evacuated Medium
22 on	0.5	15.58	1.83	2.1×10^{-7}
	1.0	31.09	2.28	....
	2.0	54.93	3.04	2.9×10^{-7}
	4.0	66.35	2.92	1.2×10^{-5}
	8.0	71.37	3.00	8.0×10^{-6}
22 pb	0.5	16.21	1.70	3.1×10^{-7}
	1.0	30.98	2.23	3.1×10^{-7}
	2.0	57.13	2.85	3.2×10^{-7}
	4.0	72.15	2.88	6.8×10^{-6}
	8.0	83.31	2.98	1.1×10^{-5}
22 qt	0.5	15.31	1.62	3.1×10^{-7}
	1.0	26.73	2.46	4.7×10^{-7}
	2.0	48.81	2.69	1.4×10^{-5}
	4.0	49.92	2.71	2.4×10^{-5}
	8.0	47.53	2.82	3.0×10^{-5}
22 rh	0.5	Lost	....	1.9×10^{-7}
	1.0	27.86	2.60	5.3×10^{-7}
	2.0	54.40	2.81	6.2×10^{-7}
	4.0	49.34	3.09	1.7×10^{-5}
	8.0	51.96	3.15	1.7×10^{-5}

* The medium consisted of 1 percent peptone and 0.5 percent K_2HPO_4 plus different concentrations of dextrose (10 c.c. of medium in vacuum bulbs). Incubation was maintained at 30 C.

From this experiment, it is evident that the low values of the hydrogen ion concentrations found in dilute sugar cultures of the "high ratio organisms" are not limiting values. What the limits are is yet to be accurately determined.

For the present purposes, we need not know them. The only point with which we now are concerned is that in dextrose concentrations of 1 percent or lower, and in the presence of sufficient protein, dibasic phosphate, or similar "regulator," the limiting hydrogen ion concentration is not reached in "high ratio cultures."

The next point to decide is how far we may lower the sugar content and still allow a sufficient amount for the attainment of maximal acidity in "low ratio cultures." The exact minimum we have not determined, but it will appear later that 0.5 percent dextrose in a medium containing 0.5 percent peptone and 0.5 percent K_2HPO_4 is safely above the minimum.

The next point to which attention must be called is this: If an organism of either class be cultivated in a medium containing sufficient "regulator" to prevent a great rise in hydrogen ion concentration with small increases in the acid content, and if the sugar content is then so low that the limiting hydrogen ion concentration cannot be reached, the medium under aerobic conditions will become more alkaline after the exhaustion of the sugar.

This is shown admirably in Chart 1. In this experiment, a "low ratio organism" (22hx) was cultivated at 37.5 C. in media containing 1 percent Witte peptone and varying percentages of dextrose. The cultures were held in Jena test tubes. From time to time a tube of each of the six media was cooled to 30 C. and its hydrogen ion concentration measured electrometrically. In plotting the results, the hydrogen ion concentrations have been laid off as ordinates against days as abscissae.

Similar results are obtained in other media with both high and low ratio organisms, but, with a high ratio culture, in order to prevent a reversion of the reaction, the dextrose content must be raised considerably above 1 percent.

As a result of these experiments, we concluded that in a medium consisting of 0.5 percent Witte peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4, the "low ratio cultures" would attain their maximal hydrogen ion concentration without a reversion; while the "high ratio organisms" would not attain a limiting hydrogen ion concentration and consequently there would be a tendency to reversion of the reaction. That such is the case is shown in Chart 2. In this experiment, two "high ratio organisms" (pb and qt), one "low ratio organism" (hx), and one "∞ratio organism" (tj) were grown aerobically in Jena test tubes at 30 C. in media made up of 0.5 percent peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4. The hydrogen ion concentration (ordinates) is plotted in Chart 2 against hours (abscissae). Between the twenty-eighth and fiftieth hours, the "low" and "∞ratio cultures" were still rising, while the "high ratio cultures" had begun to decline. Then from the fiftieth hour all but the "low ratio culture" declined. The "low ratio culture" had shown no signs of a reversion after 288 hours. The decline in hydrogen ion concentration is not due to loss of carbon dioxid, for the measurements were made after evacuation of the cultures with a Geryck pump. Nor should it be assumed that the reversion is due solely to an ammonia production. Rather does it appear to be due to another well-defined phenomenon, which we shall consider in another paper.

From all these experiments and several others, which it is not essential to describe, we are in a position to make a good guess that, in a medium consisting of 0.5 percent peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4, all "low ratio cultures" at the end of 3 to 4 days will show their limiting hydrogen ion concentrations, while all

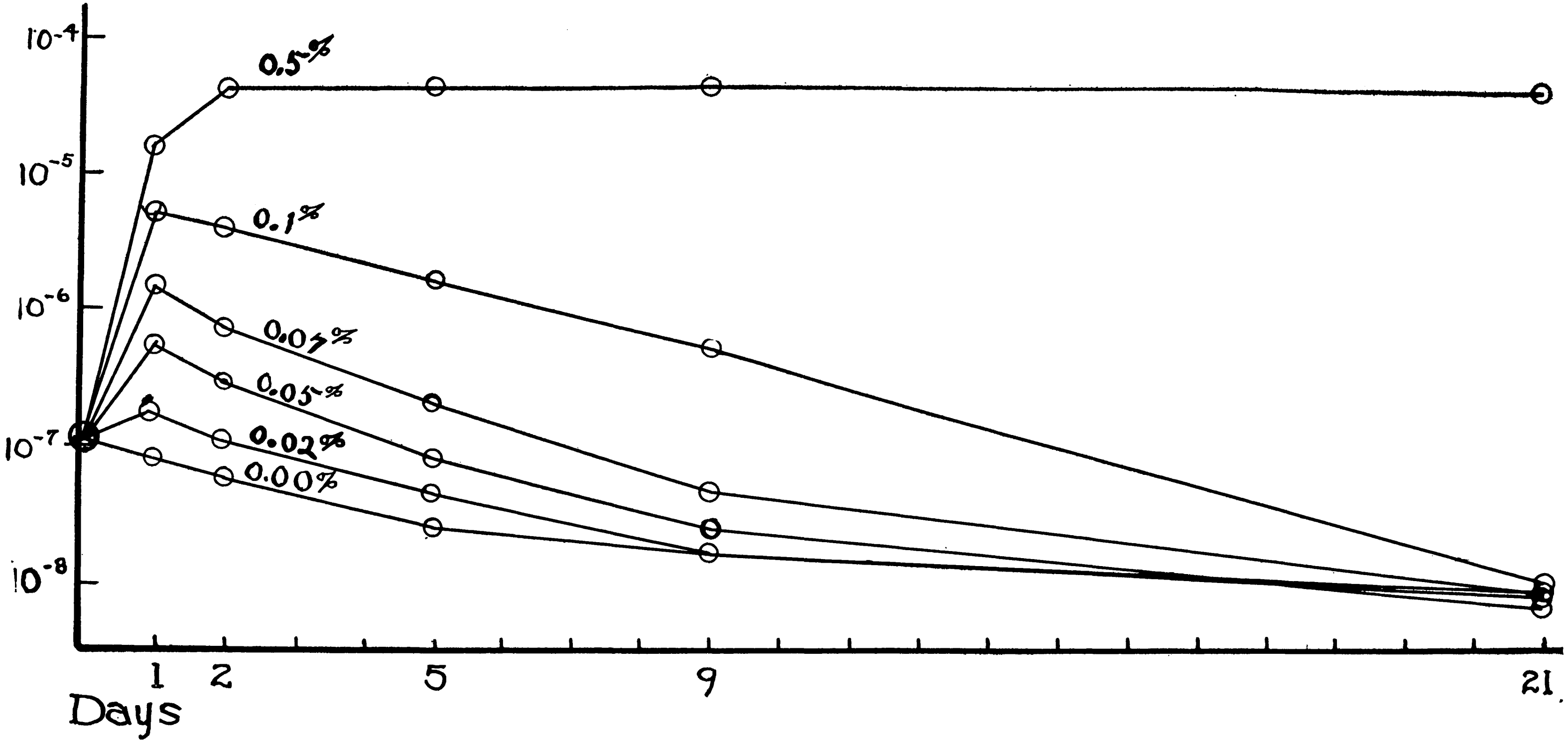

Chart 1.—Progressive change in hydrogen ion concentrations in 1 percent peptone + different percentages of dextrose. A low ratio culture (22 hx) at 30 C.

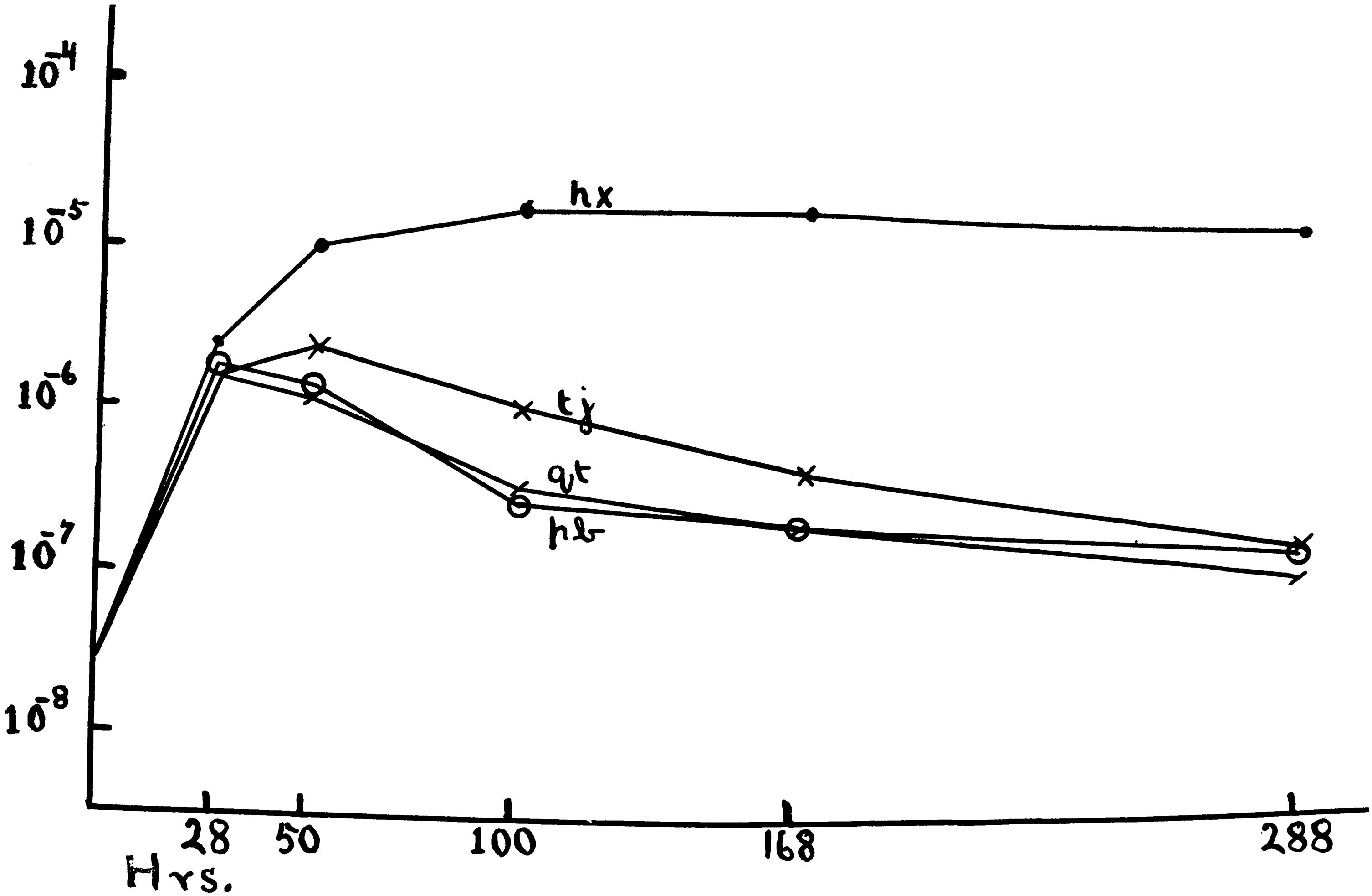

Chart 2.—Progressive change of hydrogen ion concentration in 0.5 percent peptone, 0.5 percent dextrose, 0.5 percent K_2HPO_4. The line hx represents low ratio culture; tj, ∞ ratio culture; qt and pb, high ratio cultures.

"high ratio cultures" will show a hydrogen ion concentration far below this. Even in media less favorable for the detection of such differences, as we have already shown in Table 3, the differences detected by electrometric methods are great. Some results with the "0.5 percent medium" (p-) are shown in Table 5.

TABLE 5

HYDROGEN ION CONCENTRATIONS OF LOW AND HIGH RATIO CULTURES*

Culture	Ratio	$C\overset{+}{H}$	Cultures	Ratio $CO_2 : H_2$	$C\overset{+}{H}$
hx	1.03	2.0×10^{-5}	qw	2.23	1.0×10^{-7}
st	1.06	1.0×10^{-5}	rr	2.24	2.2×10^{-7}
oo	2.39	3.6×10^{-7}	rs	2.44	5.4×10^{-7}
pb	2.23	5.9×10^{-7}	ru	2.37	2.0×10^{-7}
pg	2.44	5.1×10^{-7}	rx	2.50	5.1×10^{-7}
pj	2.92	1.3×10^{-7}	ry	2.60	7.3×10^{-7}
po	2.23	7.8×10^{-7}	sa	2.78	5.1×10^{-7}
pw	2.39	0.1×10^{-7}	sd	2.08	2.3×10^{-7}
py	2.36	3.5×10^{-7}	so	2.12	1.5×10^{-7}
qb	2.42	0.5×10^{-7}	tc	2.37	4.3×10^{-7}
qr	2.34	0.7×10^{-7}	tu	2.82	4.4×10^{-7}

* The medium consisted of 0.5 percent Witte peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4 in Jena test tubes (aerobic growth). Incubation was maintained at 30 C. for a period of 3-5 days.

To detect differences of such magnitude, it is not necessary to use the hydrogen electrode. The well-known colorimetric method is applicable, even without the use of comparison solutions of known hydrogen ion concentration.

In selecting a proper indicator for this purpose, we must choose one which, unlike the majority of indicators, will give correct indications of hydrogen ion, concentration in the presence of peptone. Two such indicators which may be used in the zone of hydrogen ion concentration with which we are concerned have already been studied. They are paranitrophenol, which Sørenson[6] has used with good effect, and methyl red, which Palitzsch[7] found to be reliable.

Paranitrophenol exists as a colorless compound in solutions with a hydrogen ion concentration above 1×10^{-5}N. In solutions of lower hydrogen ion concentration, it gradually becomes colored until at 1×10^{-6} it is a brilliant yellow green. By the use of comparison solutions of known hydrogen ion concentration, it is possible to make a very accurate estimation of the hydrogen ion value of a medium if it falls within this zone. This more exact method has not been found necessary in our procedure.

In the same way, methyl red is useful in approximately the same zone and its color changes are much more varied and brilliant, ranging from a brilliant red above 1×10^{-5} to a clear yellow below 1×10^{-6}. The color changes are so brilliant that we recommend this indicator in preference to paranitrophenol.

CULTURES TESTED

Before proceeding to a description of the results obtained with the indicator test, it will be well to classify the cultures used.

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[6] Compt. rend. trav. de Lab. Carlsberg, 1909, 8, p. 1.

[7] Ibid., 1911, 10, p. 162.

Of the organisms isolated from milk and milk products and described in the first paper by Rogers, Clark, and Davis,[2] there were available only 22 s and 22 aj, both "high ratio cultures." In this same paper, 22 fg, a "low ratio organism" from human feces, was described. This was available.

Of the organisms isolated from bovine feces and described by Rogers, Clark, and Evans,[3] there were available seventeen of the "low ratio cultures," and the one "high ratio culture."

Of the cultures isolated from grain and described by Rogers, Clark, and Evans in their second paper,[3] there were 155 "high ratio cultures," 8 "low ratio cultures," and 7 "∞ ratio cultures." All were available. We had also another "low ratio organism." Besides these cultures, we had six sent to this laboratory by Dr. Kligler and described as atypical members of the colon family. The gas determinations with these cultures are given in Table 6. The methods and special medium employed were those described in the papers by Rogers, Clark, and Evans.[3]

TABLE 6

GAS DETERMINATIONS WITH KLIGLER'S "ATYPICAL CULTURES"

Kligler's Number	Dairy Division Designation	Total Gas in c.c	Ratio CO_2: H_2
451	22 ol	27.18	2.40
452	22 oh	16.72	1.47
453	22 ok	6.87	1.06
454	22 of	18.27	2.28
179	22 og	0.91	CO_2 and H_2 present
370	22 oj	8.02	∞

So far as gas analyses indicate, three of these cultures agree very well with typical cultures of our own collection. This can best be shown by tabulating the data under consideration with the averages of our own cultures as is done in Table 7.

TABLE 7

COMPARISON OF GAS DETERMINATIONS IN ATYPICAL CULTURES WITH TYPICAL CULTURES

Organism	Total Gas c.c.	Ratio CO_2: H_2
451 — 22 ol	27.18	2.40
Average of high ratio cultures, grain series	29.50	2.36
453 — 22 ok	6.87	1.06
Average of low ratio cultures, feces series	14.09	1.06
Average of low ratio cultures, grain series	12.41	1.06
370 — 22 oj	8.02	∞
Average of ∞ ratio cultures, grain series	5.50	∞

If, then, we classify these three of Kliger's six cultures as typical, it leaves in our collection three which may be described provisionally as atypical. We understand that Kligler's cultures were from the collection of the American Museum and had probably been carried upon artificial media for a long time.

In summary, we had 20 "low ratio cultures," 159 "high ratio cultures, 8 "∞ ratio cultures," and the three "atypical" strains, which we have not yet extensively studied.

PRELIMINARY TESTS

When the time arrived for the gas determinations with the grain cultures, enough was known of the basis of the method which we are here describing to induce us to test the medium after the collection of the gas. In every case, the "low ratio cultures" were acid to paranitrophenol, and the "high ratio cultures" alkaline. All the "∞ ratio cultures" in this experiment were acid.

To see whether the same differentiation would hold under the simpler aerobic conditions of test tube culture, we tested all these grain cultures again. In this experiment, the medium was that employed in the gas determinations, except that the peptone was lowered to 0.5 percent to gain a solution of less color. Again all the "low ratio cultures" at the end of a week were acid to paranitrophenol, while all the "high ratio cultures" were distinctly alkaline, except four. Of the "∞ ratio organisms," three were distinctly alkaline, one distinctly acid, and three doubtful. As we shall see, the modified medium, developed for the final tests, eliminated the exceptions among the high ratio organisms.

FINAL TESTS

With the medium modified in accordance with the principles discovered in developing the method, we tested all the cultures available. Since the methods used are those we can confidently recommend, they will be described in detail.

Medium.—0.5 percent Witte peptone, 0.5 percent dextrose, 0.5 percent K_2HPO_4. To prepare 1,000 c.c. of this, mix 5 gm. Witte peptone, 5 gm. chemically pure dextrose, and 5 gm. of K_2HPO_4 in 800 c.c. distilled water. Heat with occasional stirring for twenty minutes over steam and filter through a Schleicher and Schüll No. 588 folded filter. Cool to 20 C. and make up to exactly 1,000 c.c. with distilled water. Tube in approximately 10 c.c. portions in clean, sterile test tubes. Sterilize by the intermittent method.

Materials.—If possible, select a sample of Witte peptone giving solutions of least color. Use only the purest dextrose. It is absolutely essential that the potassium phosphate should be chemically pure K_2HPO_4. Several samples that we have received, which were labeled K_2HPO_4 by reliable dealers, have been found to be KH_2PO_4, or mixtures of mono and dibasic phosphates. The test tubes used should be either old but unetched tubes of ordinary glass or, preferably, those made of Jena glass and steamed before sterilization.

Incubation Temperature.—30 C.

Incubation Period.—At least three days; five days is recommended.

Paranitrophenol Solution.—Dissolve 0.2 gm. re-crystallized paranitrophenol in 30 c.c. alcohol and dilute to 500 c.c. with distilled water.

Methyl Red Solution.—Dissolve 0.1 gm. methyl red in 300 c.c. alcohol and dilute to 500 c.c. with distilled water.

Procedure in Testing Cultures.—Pour about 5 c.c. of the 10 c.c. of culture into a clean, clear glass test tube and add 2-4 drops of paranitrophenol to one portion and 1-2 drops methyl red to the other. Record the color change. If the color change in any case is doubtful, compare the tint with those of a set of tubes containing solutions of known hydrogen ion concentration. For the preparation of such solutions, see the monograph by Michaelis.[8]

We have not found the use of such standards necessary, but we recommend their use if the range of color changes of the indicators is not familiar.

With the method here outlined, we tested all cultures available, of which we had 20 low ratio, 159 high ratio, 8 ∞ ratio, and three "atypical" strains. In the first test paranitrophenol was used. All the "low ratio cultures" were distinctly acid. All the "high ratio cultures" were distinctly alkaline. All the "∞ ratio cultures" were distinctly alkaline. Of the "atypical" cultures 179-22og and 452-22oh were acid, while 454-22of was alkaline.

The six Kligler cultures were again tested and agreed as before, except that 451-22ol, which is a distinct high ratio organism, failed to react distinctly alkaline once in three trials. In this case, its hydrogen concentration was 7.8×10^{-6}, a reaction easily distinguished with methyl red from the brilliant color given by a low ratio culture.

In order to check these results, the series of 190 cultures were again tested. This time the tests were made by one of us who knew the individual history of only two of the cultures and who consequently was not prejudiced in his judgment. The incubation period was also shortened to three days, and the applicability of methyl red was tested. Without exception, the "low ratio cultures" were perfectly colorless to paranitrophenol and brilliantly red to methyl red. Without exception, the "high ratio cultures" were distinctly colored with paranitrophenol. All but five were distinctly yellow to methyl red. These five were in the neutral tints, but so far below the distinct and brilliant red given by the "low ratio cultures" that there was no difficulty whatever in distinguishing them. Of the "∞ ratio cultures," two were alkaline and six acid to both indicators. Of the atypical cultures 454-22of was alkaline, and 452-22oh and 179-22og were acid to both indicators.

The "∞ ratio cultures," it will be noticed, reacted acid to paranitrophenol in the preliminary test under anaerobic conditions. In the second aerobic preliminary test, part were alkaline, part acid. Reducing the sugar content and allowing five days' growth in the first "final" test brought them all to an alkaline reaction. In the last experiment, three days' growth left some alkaline and others acid. We have a plausible explanation of this inconsistency, not based entirely upon the results shown in Chart 2, but well illustrated by those curves. Inspection of these curves shows that the "∞ ratio culture" rose to a higher point than either of the "high ratio cultures." Unlike the "low ratio cultures," however, it found 0.5 percent dextrose in this particular medium insufficient for the attainment of a limiting acidity. Consequently, a reversion took place. Had the oxygen supply been shut off, as it was in the first experiment, the reversion would not have taken place. But even under aerobic conditions, as in the second experiment, 1 percent of sugar allowed the production of so much acid that even seven days did not suffice to revert the hydrogen ion concentration to a point alkaline to paranitrophenol. Reducing the sugar content and allowing five days' activity brought all the cultures to a point alkaline to paranitrophenol, but the three days' growth in the last experiment was not sufficient for an extensive reversion.

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[8] Das Wasserstoffionen-Konzentration, 1914.

Were we to study the conduct of these "∞ ratio organisms" in more detail, it is highly probable that a very simple method could be devised for distinguishing them from the "high" and "low ratio cultures." This, however, is hardly necessary at present, since other cultural characteristics, such as their peculiar growth on agar, are sufficient to prevent their being mistaken for true members of the colon-aerogenes family.

In regard to the three "atypical" cultures, we shall draw no conclusions at present.

On the other hand, the results with the "high" and "low ratio cultures" leave no doubt that a set of very simple conditions has been established which permits a differentiation of these two groups by a very simple test. In all the cases tested under anaerobic conditions, the correlation between gas ratio and indicator test was perfect. In the medium modified to suit simpler cultural methods under aerobic conditions, the correlation was again perfect.

At present we know of no single test which correlates with the gas ratio to this extent. We have "high" and "low ratio cultures" which produce indol, liquefy gelatin, produce similar pigments, and ferment the same carbohydrates and their derivatives; but a close study of the gas-producing powers of these two classes reveals striking differences. Accurate estimations of their acid-producing and acid-resisting powers reveal striking differences. We believe that in these differences in the gas ratio, on the one hand, and in the acid production, on the other, we have indices of fundamental differences in metabolism.

With these differences established, it only remained to find the conditions and the means for making them evident to the eye. Then a simple diagnostic test resulted. Its value lies in the established correlation between the physiologic characteristics it detects, and the sources of the organisms possessing these characteristics. Its reliability rests, not only upon the established correlation with the gas ratio, but upon the fact that it is based upon somewhat firmer foundations than the chance observation of some minor physiologic peculiarities.

But with all this in its favor, a final word of caution is necessary. The three "atypical" strains studied, we have not yet classified by either gas ratio or indicator test. It is highly probable that very old cultures, such as these were, or strains found in nature will have "atypical" properties and will frequently occur in extensive collections. Judgment of these requires the greatest caution, and the application to such cultures of any biochemical test, such as the one we have outlined, unless it be applied with sufficient care to give it the dignity of the most accurate quantitative measurement available, can hardly be considered worthy of serious consideration.

Having emphasized this caution, we feel justified in urging that the method here developed be subjected to careful investigation by others and in connection with accurate gas determinations. If it differentiates the cultures in other collections of the colon-aerogenes group as well as it has ours, it should prove a useful method.

Since one of the requisites of a routine method seems to be rapidity, it may be well to note that exclusive of the time required for the preparation and inoculation of the tubes, fifty tests can be made in an hour. The incubation period may seem long to those impatient for results, but we urge a five-day period at 30 C. with no apology.

Many applications of the general principles involved in this method have already suggested themselves, and work upon some of these is now in progress.

We seize this opportunity to express our appreciation of Mr. Rogers' help in caring for the cultures and in making the large number of inoculations required.

SUMMARY

The diagnostic value of the gas ratio and the sanitary significance of the correlation between gas ratios and the sources of the organisms of the colon-aerogenes family studied in this laboratory have suggested the utility of a simple test which will distinguish the two main groups.

By the use of the hydrogen electrode, the changes in hydrogen ion concentration have been followed in various cultures of the bacteria in question. By such studies differences in the metabolism of different species have been detected, and the correlation of these differences with the gas ratios has been established.

Simple conditions have been found under which metabolism can be so controlled that the hydrogen ion concentrations of cultures of one group can be made to diverge widely from those of the other group. These differences can be sharply distinguished by means of paranitrophenol or methyl red. In this way a simple diagnostic test has been established the results of which correlate perfectly with the gas ratios of the two main groups of the colon-aerogenes bacteria.